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Effects of intravenous infusion of *E.coli* lipopolysaccharide in early pregnant cows

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Abstract

The objective was to characterize effects of Escherichia coli LPS (given iv) on corpus luteum (CL) and embryonic viability in early pregnant cattle. Eight non-lactating German Holstein cows were given 0.5 µg/kg LPS on 35 ± 3 d (mean ± SEM) of pregnancy, whereas seven heifers, 41 ± 6 d pregnant, were given 10 ml saline (Control group). Transrectal B-mode examinations of the CL were done at -1, 3, 6, 12, 24, 48, 72, and 96 h relative to treatment. Blood samples were collected at -1, 0.5, 1, 2, 3, 4, 6, 9, 12, 24, 48, 72, and 96 h. At 12 and 48 h, the CL was biopsied. None of the cows still in the experiment 10 d after LPS (n=7) had embryonic loss. In LPS-treated cows, luteal area decreased (from 4.1 to 3.1 cm2; P≤0.05) within 6 h and until 48 h. Luteal blood flow decreased by 39% (P≤0.05) within the first 6 h after LPS, but returned to pre-treatment values by 48 h. Plasma P4 decreased by 62% (P≤0.05), reached a nadir (2.7±0.6 ng/mL) at 12 h after LPS and was not restored to pre-treatment (P≤0.05). In luteal tissue, mRNAs for StAR and for FGF1 were lower (P≤0.05) in LPS- than in saline-treated cattle at 12 h, with no difference between groups at 48 h. Levels of mRNAs for Casp3 and FGF2 were not different between groups (P>0.05) at 12 or 48 h after treatment. In conclusion, LPS transiently suppressed CL function, but did not induce embryonic mortality.

Key words: pregnancy; corpus luteum; endotoxin; gene expression; Color Doppler ultrasonography; progesterone; prostaglandin; cattle

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Introduction

Embryonic mortality is of cardinal importance in bovine reproduction (Zavy 1994, Lucy 2001). Embryonic mortality is usually classified into two categories, based on the time of return to estrus. In cattle with early embryonic mortality, estrus occurs before day 24 post insemination (p.i.), compatible with embryonic death before day 16 p.i., whereas in late embryonic mortality, cattle return to estrus after day 24 p.i., apparently due to death of the embryo after day 16 p.i. (Kastelic & Ginther 1989, Humblot 2001, Santos *et al.* 2004a). Early embryonic mortality affects ~30 to 40% of all inseminated cattle, substantially reducing fertility (Thatcher *et al.* 1994, Silke *et al.* 2002, Diskin & Morris 2008). Late embryonic mortality is less frequent (5 to 20% of inseminated cattle) but causes greater economic losses per affected animal, as it substantially prolongs calving to conception intervals (Diskin & Sreenan 1980, Sreenan & Diskin 1983, Santos *et al.* 2004a).

Inflammatory processes during early pregnancy have been associated with embryonic mortality in cattle (Hansen *et al.* 2004), attributed to production of prostaglandin F2alpha ($PGF_{2\alpha}$) causing luteolysis and pregnancy loss (Manns *et al.* 1985, Stewart *et al.* 2003, Inskeep 2004). In cows given *Escherichia coli* lipopolysaccharides (*E. coli* LPS) during the first, second and third trimesters of pregnancy, 6 of 10 cows aborted (three, one and two in the first, second and third trimesters, respectively). Abortions were attributed to a prolonged release of $PGF_{2\alpha}$ (Giri *et al.* 1990) causing reductions in progesterone (P_4), especially during the first trimester (in the absence of an extraluteal source of P_4).

In a recent *in vivo* study, intravenous administration of *E.coli* LPS (model for

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inflammation) temporarily decreased CL size and function in diestrus cows (Herzog *et al.* 2012). Luteal blood flow, plasma P_4 concentration and CL size decreased within 3, 9 and 24 h, respectively, after LPS. However, estrous cycle length was unchanged. Therefore, premature luteolysis did not occur.

The aim of the present study was to investigate effects of LPS exposure on the CL and on embryonic viability in early pregnant cattle. For this purpose, peripheral P_4 was used as main assessment of luteal secretory activity (Sartori *et al.* 2004, Lüttgenau *et al.* 2011). Furthermore, the relationship between endotoxin exposure and luteal function was evaluated at a subcellular level in biopsies of luteal tissue. Molecular parameters known to yield information regarding key cellular functions of the CL were selected (Niswender *et al.* 2000). Specifically, steroidogenic acute regulatory protein (StAR) is responsible for transportation of cholesterol to the inner mitochondrial membrane, a rate-limiting step in P_4 synthesis (Stocco & Clark 1996). Furthermore, Fibroblast Growth Factors (FGFs) participate in regulation of development and regression of the CL. In that regard, acidic and basic FGFs (FGF1 and FGF2, respectively) are increased during functional luteolysis (Doraiswamy *et al.* 1998, Neuvians *et al.* 2004); FGF2 is also involved in CL formation and controls P_4 secretion (Yamashita *et al.* 2008); and during luteolysis, Caspase-3 (Casp3) has a pivotal role in selective destruction of key structural and functional cellular proteins (Casciola-Rosen *et al.* 1996, Thornberry 1998).

Materials and methods

Cattle

This study was conducted (between April 2011 and May 2012) at the Clinic for Cattle, University of Veterinary Medicine, Hannover, Germany. The experimental protocol was reviewed and approved (Lower Saxony Federal State Office for Consumer Protection and Food Safety, 33.9-42502 – 04-11/0515) and the research was conducted in accordance with German legislation on animal welfare. Eight non-lactating German Holstein cows, clinically healthy and 35±3 d (mean±SEM) after insemination, were used for the LPS trial. These cows were 3.7±0.8 y old.

In addition, seven German Holstein heifers from the FLI (Friedrich Loeffer Institut – Federal Research Institute for Animal Health) in Mariensee, Germany were used as a control group (Saline group; n=7). They were 41±6 d pregnant and 2.2±0.3 y old, younger than the LPS-treated group (P≤0.05). All cattle were tethered in stalls and given *ad libitum* access to hay and water.

Study design

The LPS group were subjected to an Ovsynch protocol (Pursley *et al.* 1995), bred by timed artificial insemination, with pregnancy diagnosis (transrectal ultrasongraphy) done ~25 d after breeding. On day 35±3 of pregnancy, a polyethylene catheter was inserted into a jugular vein. At 09.00 the following day, an LPS solution (0.5 µg/kg body weight of *E. coli*, O55:B5; L2880, Sigma Aldrich, Steinheim am Albuch, Germany) in 10 ml sterile water (B. Braun, Melsungen) was given iv (over an interval of ~1 min). After administration of LPS, the jugular catheter was flushed with 25 ml 0.9% saline solution. Control heifers were bred following detection of estrus and given 10 ml of 0.9% saline solution instead of LPS on day 41±6 of pregnancy.

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119 ***Ultrasonography for assessment of the CL and pregnancy***

120 Transrectal B-mode ultrasonographic examinations were done at -1, 3, 6, 12, 24,
121 48, 72, and 96 h relative to treatment (saline or LPS) to assess luteal size and luteal
122 blood flow. A Logiq Book XP ultrasound scanner (General Electrics Medical Systems,
123 Jiangsu, P.R. China), equipped with a 10.0-MHz linear-array transducer (General
124 Electrics Yokogawa Medical Systems, Tokyo, Japan), was used. For luteal size, three
125 cross-sectional images with maximal areas of the CL were recorded (using B-mode
126 sonography) and luteal areas were measured offline (PixelFlux®, Version 1.0,
127 Chameleon Software, Leipzig, Germany). If the CL had a cavity, the area of the cavity
128 was measured and subtracted from total area (Kastelic *et al.* 1990). Means of the cross-
129 sectional areas of the three images were calculated and used for statistical analyses.
130 For luteal blood flow, power-flow Doppler was used for color blood flow mapping of the
131 CL in various transverse sections, as described (Herzog et al. 2012).

132 To assess embryo/fetal viability, heartbeat was assessed with B-mode transrectal
133 ultrasonography at 6, 12, 24, 48, 72, 96 h and at 10 d after infusion.

134

135 ***Blood samples and determination of plasma P_4 and prostaglandin metabolites***
136 ***concentrations***

137 At 1.0 h before administration of LPS or saline solution, blood samples were collected to
138 characterize concentrations of P_4 and PGFM. In addition, blood samples were collected
139 (via the catheter) at 0.5, 1, 2, 3, 4, 6, 9, 12, 24, 48, 72, and 96 h after treatment.

Samples were stored on ice (0°- 4°C), centrifuged (3000 x g, 15 min at 4°C) within 30 min after collection and plasma removed stored at -20°C pending analyses.

Serum P_4 concentrations were determined with a commercial chemiluminescence immunoassay (Immulite®, Siemens Healthcare Diagnostics, Deerfield, IL, USA). The lower detection limit was 0.5 ng/ml, and intra- and inter-assay coefficients of variation were <10%. Plasma PGFM concentrations were determined with a competitive enzyme immunoassay (Mishra *et al.* 2003). The PGFM-horseradish peroxidase conjugate and antiserum were supplied by Prof. Meyer (Physiology Weihenstephan, Technische Universitaet Muenchen, Freising, Germany), whereas PGFM used for the standard curve was purchased from Sigma, Germany. The antiserum had minimal (<0.01%) cross reactions with PGE_2, PGEM, PGA_2, PGAM, and $PGF_{2\alpha}$ (Guven & Ozsar 1993). The lowest detection limit for PGFM was 25 pg/ml. Intra- and inter-assay CVs were 3.5 and 11.4%, respectively.

Corpus luteum biopsy

Corpus luteum biopsies were done as described (Herzog *et al.* 2012) at 12 and 48 h after treatment. At each time of sampling, at least two tissue samples per animal were recovered and immediately placed in a sterile DNase- and RNase-free cryotube (Fa. Brand, Wertheim, Germany), frozen in liquid nitrogen, and stored at -80°C. This enabled repeated biopsy sampling from a single CL without impairing its function, as described (Tsai *et al.* 2001).

RNA extraction, cDNA synthesis and real-time qPCR

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Total RNA was isolated from biopsy samples using QIAzol Lysis Reagent (QIAGEN Germany, Hilden), followed by two-step quantitative real-time RT-PCR (Pistek *et al.* 2013). A Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) and RNA 6000 Nano Kit (Agilent) were used to assess RNA Quality. Mean RIN values were 6.5 (range, 5.5-8.0). The qPCR was done with a CFX384TM Real-Time PCR Detection System (Bio-Rad, München, Germany) and PCR Mix SensiFast SYBR and Fluorescein Kit (Bioline, London, UK). Primers used are shown in Table 1. The cycle of quantification (Cq) was calculated after baseline subtracted curve fitting using the single threshold method (Bio-Rad CFX Manager V1.5.534.0511 software). Relative quantification of qPCR products was done as described (Livak & Schmittgen 2001). Geometric means of reference genes *H3F3A, UBK3, YWHAZ* were used for normalizing mRNA expression of target genes *FGF1, FGF2, StAR, and Casp3,* according to the BestKeeper method (Pfaffl *et al.* 2004).

Statistical analyses

For age, luteal size and blood flow, uterine blood flow parameters, and concentrations of P_4 and PGFM, residuals within groups were visually examined for normal distribution (PROC CHART) and analysed with a Shapiro-Wilk test (PROC UNIVARIATE). All statistical analyses were done with SAS® (version 9.1, Statistical Analysis Institute Inc., Cary, NC, USA).

Residuals within groups for the variable age did not differ significantly from a normal distribution. Hence, an independent two-sample Student's *t*-test (PROC TTEST) was used for comparison of the mean ages of the two groups.

Residuals within groups for variables LBF, *FGF1*, *FGF2*, *StAR* and *Casp3* also did not differ significantly from a normal distribution. Hence, these variables were submitted to a two-factorial variance analysis for repeated measurements (PROC GLM). The influence of various time points within groups was tested using repeated measurements variance analysis (PROC GLM). A Tukey's HSD test was used to locate differences between groups. A Student's *t*-test for paired samples (PROC MEANS) was used to compare measurement times within a group. Since variables P_4, PGFM and LS did not have a normal distribution, nonparametric tests were used. Effects of measurement time on P_4 and LS were submitted to a Friedman's Test (PROC SORT) and differences between groups were located using Wilcoxon's rank sum test (PROC NPAR1WAY). Differences between time points within a group were tested using a Wilcoxon's signed rank test (PROC UNIVARIATE). For all statistical tests, a significance level of $P \leq 0.05$ was specified. Values for LBF, *FGF1*, *FGF2*, *StAR* and *Casp3* are expressed in text, figures and tables as mean ± standard deviation (SD). For P_4, PGFM and LS, median and median absolute deviation (MAD) were used.

Results

Clinical response

Treatment with LPS induced various toxemia-related clinical signs, including tachycardia, tachypnea, dyspnea, epiphora and mild diarrhea, as well as a slight increase of body temperature (maximal 39.7°C). However, all but one of the cattle were clinically normal by 12 h after treatment (Table 2). One cow was found dead 24 h after LPS treatment, with death attributed to cardiovascular failure. There were ~5 L of clotted

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blood in the abdominal cavity, likely due to hemorrhage from the CL biopsy site, that certainly contributed to her demise. There was no pregnancy loss during or up to 10 d after LPS treatments (≤12 h: n=8; >12 h: n=7) and Control heifers did not have any clinical manifestations of toxemia.

Luteal area and luteal blood flow

Luteal size (LS) was 12% smaller (P≤0.05) at 6 h after LPS treatment, reached a nadir (3.1±0.3 cm²) at 12 h, remained low until 24 h, and then returned to pre-treatment values by 48 and thereafter remained unchanged (Fig. 1). For luteal blood flow (LBF), there were effects of treatment, time, and a treatment by time interaction (P≤0.05; Fig. 1). In LPS-treated cows, LBF decreased by 39% (P≤0.05) at 6 h, reached a nadir (0.9±0.2 cm^2) at 12 h, and then rebounded to pre-treatment values by 48 h (Fig. 1). In Control heifers, LS and LBF remained relatively stable.

Plasma P_4 and PGFM concentrations

For plasma P_4 concentrations, there were effects of time (P≤0.05) and a treatment by time interaction (P≤0.05; Fig. 2). Plasma P_4 concentrations increased from 7.1±0.7 to 10.3±2.7 ng/ml (median±MAD; P≤0.05) within the first 30 min after administration. Thereafter, P_4 decreased between consecutive measurements (P≤0.05) beginning 4 h after infusion, reached a nadir (2.7±0.6 ng/ml) 12 h after infusion, increased again between 24 and 48 h (P≤0.05), but was not restored to pre-treatment values. In Control heifers, P_4 concentrations did not vary significantly between consecutive measurements during the first 12 h (P>0.05). Progesterone concentrations decreased (P≤0.05) between

12 and 24 h after saline infusion, increased again between 24 and 48 h (P≤0.05) and thereafter remained constant. Plasma P_4 concentrations were higher in LPS-treated than control cattle within the first 30 min after treatment (P≤0.05), lower (P≤0.05) in LPS-treated cattle between 9 and 12 h after treatment and higher (P≤0.05) in LPS-treated cattle at 48 h.

For PGFM, there were effects of time, and a treatment by time interaction (P≤0.5; Fig. 2). Plasma PGFM concentrations increased almost by an order of magnitude within the first 30 min after infusion of LPS, but had returned to baseline by 4 h and remained there. In Control heifers, PGFM did not change significantly over time. Concentrations of PGFM were higher (P>0.05) in LPS versus Control cattle for the first 4 h after infusion.

Gene expression

In cows given LPS, transcript abundance of mRNA encoding *Casp3* was higher at 12 versus 24 h after treatment (P≤0.5, Fig. 3), whereas expression of *StAR* mRNA was lower (P≤0.5, Fig. 3) at 12 versus 48 h. There was no difference within the LPS group for *FGF2* (P>0.05), whereas *FGF1* was lower (P≤0.05, Fig. 3) at 12 versus 48 h after LPS infusion. Within the Control group, expression of mRNA did not vary between time points (P>0.05) for any parameter. Levels of mRNA encoding *StAR* and *FGF1* were lower in LPS- than in saline-treated cattle at 12 h after treatment (P≤0.05; Fig. 3) but not at 48 h. There were no differences between treatment groups for *Casp3* or *FGF2* (P>0.05).

Discussion

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In early-pregnant cows in the present study, iv administration of LPS endotoxin temporarily suppressed CL function and structure. Luteal size, LBF, and P_4 decreased within 12 h after LPS treatment, but complete luteal regression did not occur. Similarly, in a previous study, administration of LPS to diestrous cows reduced LS, LBF and P_4 during the first 24 h after treatment (Herzog *et al.* 2012), but did not cause luteal regression.

In the present study, all fetuses still in the experiment at the time (n=7) had a heart beat 10 d after treatment. Progesterone is essential for maintenance of pregnancy in cattle (Estergreen *et al.* 1967). In the first trimester of gestation, removal of the CL causes abortion. Progesterone of placental origin can support pregnancy at ~150 to 250 d of gestation in the absence of a CL. However, removal of the CL after 250 d results in calving in 1 or 2 d (Stabenfeldt *et al.* 1970, Mann & Lamming 1999, Taverne 2001, Breukelman *et al.* 2005). Furthermore, pregnancy is maintained by exogenous P_4 in cattle in absence of endogenous P_4, e.g. with the CL removed by ovariectomy (Kesler 1997) or administration of luteolytic doses of $PGF_{2\alpha}$ (Ferguson *et al.* 2014). In particular, based on a recent report (Ferguson *et al.* 2014) the sooner P_4 supplementation starts after $PGF_{2\alpha}$, the more likely the pregnancy will be maintained, with a positive outcome most likely if exogenous P_4 is given within 12 h after $PGF_{2\alpha}$. Furthermore, those authors observed that P_4 decreased fastest between 12 and 24 h after $PGF_{2\alpha}$, and was below 2 ng/ml by 30 h post-$PGF_{2\alpha}$. In this study, P_4 in LPS-treated cows was also supressed for more than 12 h in a similar pattern, but consistently exceeded 2 ng/ml. Luteal regression and declines in plasma P_4 to < 2 ng/ml after administration of exogenous $PGF_{2\alpha}$ are well established (Lauderdale 1975, Sioan 1977, Wright & Kiracofe 1988). We concluded that

277 the LPS-induced release of endogenous $PGF_{2\alpha}$ impaired luteolytic function but did not
278 cause luteolysis, thereby reducing P_4 production, but not getting systemic P_4
279 concentrations low enough to terminate pregnancy.

280 Administering viable bacteria or prolonged infusions of LPS activate the
281 immunological cascade, disrupt neuroendocrine activity (mainly by blocking or delaying
282 the LH surge), and can shorten the estrous cycle in postpartum cattle (Williams *et al.*
283 2008). Also, studies using mastitis as a model of inflammation instead of experimentally
284 LPS administration have implicated infectious diseases as causing embryonic loss (Soto
285 *et al.* 2003, Santos *et al.* 2004b). However, in the present study, LPS was administered
286 over a short interval (~1 min). Therefore, the insult was short-lived. Consequently, only
287 one PGFM peak was detected; although it clearly suppressed P_4, it did not cause
288 luteolysis and the CL subsequently recovered. In heifers, intrauterine infusions of
289 exogenous $PGF_{2\alpha}$ caused a PGFM pattern with several pulses, similar to spontaneous
290 luteolysis (Ginther *et al.* 2009). That the single PGFM peak detected in the present study
291 did not induce luteolysis was further evidence that sequential PGF pulses are crucial for
292 complete luteolysis in cattle (Ginther *et al.* 2009, Ginther *et al.* 2010).
293 In third-trimester cows (n=3, 190 to 200 d of gestation) given LPS (0.5 µg Salmonella
294 typhimurium LPS/kg) intravenously, the LPS was rapidly cleared and there were no
295 indications that it crossed the placenta. However, all three cows aborted within 10 d after
296 LPS treatment (Foley *et al.* 1993). In the present study, none of the cows aborted.
297 Perhaps the third-trimester fetus is more susceptible to LPS than cattle at ~6 wk of
298 pregnancy. Also, differences in source and dose of LPS could have contributed to
299 disparate outcomes. Effects of two doses of LPS (1.0 or 2.5 µg *E. coli* LPS/kg) in cows

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at various stages of pregnancy have been reported (Giri *et al.* 1990). In that study, LPS was infused over 6 h (which better represented exposure to LPS in natural infection), in contrast to the single bolus injection given in the present study. Furthermore, magnitudes of changes in P_4, PGFM, cortisol and other metabolites, as well as severity of clinical signs, were all dose-dependent. Although the type of LPS used was the same as in the present work, both doses were higher. Intensity of systemic responses to LPS in cattle is dose-dependent, and can considerably vary from cow to cow (Jacobsen *et al.* 2005).

Progesterone concentrations after LPS treatment in the present study were similar to those in cyclic cows (Herzog *et al.* 2012). In both experiments, P_4 peaked 30 min after LPS exposure. This was attributed to an acute release of P_4 from the adrenal gland, as in the study by Battaglia *et al.* (1997), with a nadir in P_4 after 12 h. However, P_4 concentrations in cyclic LPS treated cows remained decreased for up to 48 h after LPS exposure (Herzog *et al.* 2012), whereas the difference between early pregnant LPS and control cattle had disappeared by 24 h after LPS administration. Progesterone concentrations also recovered much faster in pregnant cows of the present study than in cyclic cows (Herzog *et al.* 2012). Furthermore, P_4 concentrations were higher 48 h after treatment in LPS-treated cows compared to controls. Similarly, for LBF, there was no difference between LPS and saline-treated pregnant cattle in LBF 24 h after LPS administration, and LBF was higher in LPS-treated cows 48 and 72 h after treatment, consistent with a robust association between P_4 and LBF, as reported (Herzog *et al.* 2010). Changes in luteal size caused by LPS infusion were less prominent in pregnant than in cyclic cows. Luteal size sharply decreased in pregnant cows, reached a nadir at

12 h and returned to initial values by 48 h after infusion, whereas in cyclic cows, the nadir occurred after 24 h and LS never regained pre-treatment baseline (Herzog *et al.* 2012).

Luteal gene expression confirmed that the CL of LPS-treated cows in the present study underwent partial luteolysis. As in cyclic cows (Herzog *et al.* 2012), *StAR* was reduced 12 h after LPS administration in pregnant cows. Already at 48 h, however, there were no differences between LPS and control cattle in expression of *StAR* mRNA. Unfortunately, in the previous experiment, expression of mRNA in cyclic cows was not assessed 48 h after infusion. Regardless, it is noteworthy that plasma P_4 concentrations differed between groups for only a short interval.

There were no significant differences between LPS-treated versus Control cows for mRNA expression of *Casp3*. Conversely, in cyclic cows (Herzog *et al.* 2012) expression of this protein was increased 12 h after administration in LPS-treated cows compared to controls, in accordance with decreased luteal size. Perhaps Casp3 has an important role in apoptosis and/or subsequent regeneration of cycling, but not in CL of pregnant cows, where it may have a lesser effect. Observations in laboratory animals support this assertion. In that regard, Casp3 can be dispensable or nonspecific in mice (Kuida *et al.* 1996, Jänicke *et al.* 1998). Furthermore, in postpartum rats, Casp3 only regulated regression of the CL generated after parturition, but had no apparent role in luteolysis of a CL formed during pregnancy (Takiguchi *et al.* 2004). Further investigation is needed to determine the relevance of these findings for the bovine CL. In addition, we evaluated Casp3 only at mRNA level. Assessing the presence of the Casp3 protein would provide

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345 further information (Johnson & Bridgham 2000, Fenwick & Hurst 2002); therefore, it
346 should be considered in future studies.

347 There were significant differences in expression of *FGF1* but not *FGF2* after LPS
348 exposure. Similarly, in a previous study, *FGF1* was reduced in cyclic LPS treated cows
349 12 h after exposure (Lüttgenau *et al.* 2016). Both FGF1 and FGF2 are regarded as
350 cellular “survival factors” (Renaud *et al.* 1994), conferring protection against apoptosis.
351 They promote angiogenesis in the developing CL, as well as support pre-existing
352 vasculature in the mature and gravid CL (Zheng *et al.* 1993, Schams 1994). Therefore,
353 reductions in *FGF1* and a corresponding decrease in LBF at 12 h after LPS infusion
354 were expected. In cyclic cows, both *FGF1* and *FGF2* were increased at 12 h after
355 induction of luteolysis (Neuvians *et al.* 2004). In contrast, in the present study, *FGF2*
356 expression did not change over time and *FGF1* expression was lower 12 h after LPS
357 infusion. Furthermore, *FGF2* was increased in diestrous cows given LPS (Lüttgenau *et*
358 *al.* 2016). Since the cited experiments were done in cyclic cattle, apparent differences
359 may have been due to pregnancy status. There may be luteoprotective mechanisms in
360 the CL of pregnant cattle that make them less susceptible to endogenous PGF,
361 consistent with the ‘rebound’ in CL function in LPS-treated cows in the present study.

362 The LPS-treated cows underwent an OvSynch protocol and timed artificial
363 insemination, whereas heifers in the control group were inseminated after estrus
364 detection. This difference was due to organizational reasons, but is potentially relevant.
365 Timed artificial insemination is a potential risk factor for late embryonic loss, although
366 direct evidence is missing (Santos *et al.* 2004a). However, rather than luteal or uterine
367 events, follicular development seems to play a relevant part in the mechanism by which

368 timed artificial insemination affects pregnancy rate (Jordan *et al.* 2009, Bollwein *et al.*
369 2010). Fortunately, none of the LPS-cows in our study underwent embryonic loss.
370 Due to organizational reasons, there was also a difference in parity number and
371 age between LPS- and Control group (primiparous cows vs. nulliparous heifers).
372 However, none of the parameters measured (LS, LBF, P_4 and PGFM) differed between
373 the two groups before LPS infusion. Furthermore, the minimal reaction to the NaCl
374 infusion of the heifers in this study resembled that of cycling cows (Herzog *et al.* 2012).
375 In addition, the Control group was mainly designed to exclude any influence of stressors
376 (e.g. manipulations related to treatment, sonography and other examinations), since, in
377 general, stress impairs reproductive function (Sheldon *et al.* 2014, Endo *et al.* 2017). In
378 this regard, there is evidence that heifers might be even less stress-resistant than cows.
379 When cattle are repeatedly exposed to stressors such as painless restraint, transport
380 and manipulation, their reaction to those stimuli progressively decreases. This applies to
381 behavioral alterations as well as to hormonal response, e.g. cortisol level (Boissy &
382 Bouissou 1988, Lay Jr *et al.* 1996, Grandin 1997, Rushen *et al.* 2001). Because of their
383 younger age, nulliparous heifers are obviously less accustomed to human contact and
384 manipulations than cows. Thus, they are likely more stress-sensitive than older cows
385 (Lay Jr *et al.* 1996). An inverse relation between parity number and intensity of reaction
386 to stressors has been reported in other species, including gestating sows (Zhang *et al.*
387 2017).
388 One cow died between 12 and 24 h after LPS infusion. At necropsy, there were
389 approximately 5 L of clotted blood in the abdomen, apparently derived from luteal biopsy
390 sites. The ultrasound-guided technique described by (Tsai *et al.* 2001) allows repeated

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biopsies from a CL, without apparent effect on its function. To our knowledge, this is the first case reporting hemorrhage from the biopsy site after using this technique. It is known from human medicine that leukopenia and thrombocytopenia develop in immunocompetent patients during septic shock (Stephan *et al.* 1997). In a healthy cow, the loss of 5 liters of blood would not cause death (Radostits *et al.* 2000). However, in an animal that was already compromised, this may have acted in a synergistic manner with acute phase symptoms, resulting in death.

In summary, administration of LPS to early pregnant cows caused a temporary depression of luteal function, but all pregnancies were preserved. Furthermore, the impact on CL was less pronounced than in a similar study involving diestrous cows. In particular, analysis of molecular parameters suggested differences in luteolytic mechanisms between the CL of the cycle and the CL of pregnancy, consistent with luteoprotective changes in the latter.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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631 **Figure legends**

632

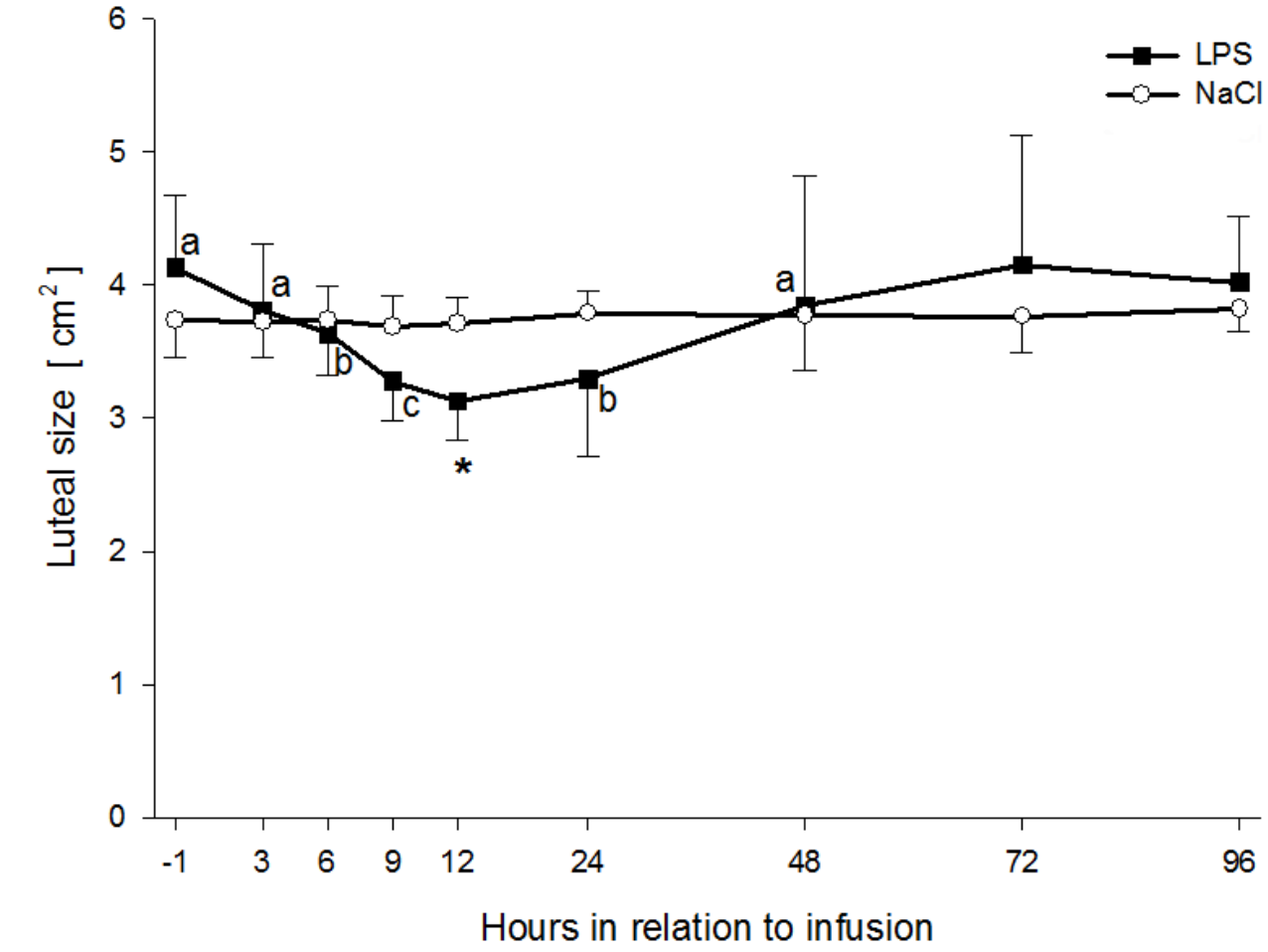
633 **Fig. 1:** Median ± MAD of luteal tissue area (Panel A) and mean ± SEM of luteal blood
634 flow (Panel B) in early pregnant cattle treated with *E. coli* lipopolysaccharide (LPS; ▪; -1
635 to 12 h: n=8, 24 to 96 h: n=7) or Saline (○; n=7), respectively.
636 *Values differed between *LPS- and Saline-treated* cattle (P ≤ 0.05).
637 [a,b]Within *LPS-treated* cattle, values without a common superscript differed (P ≤ 0.05).

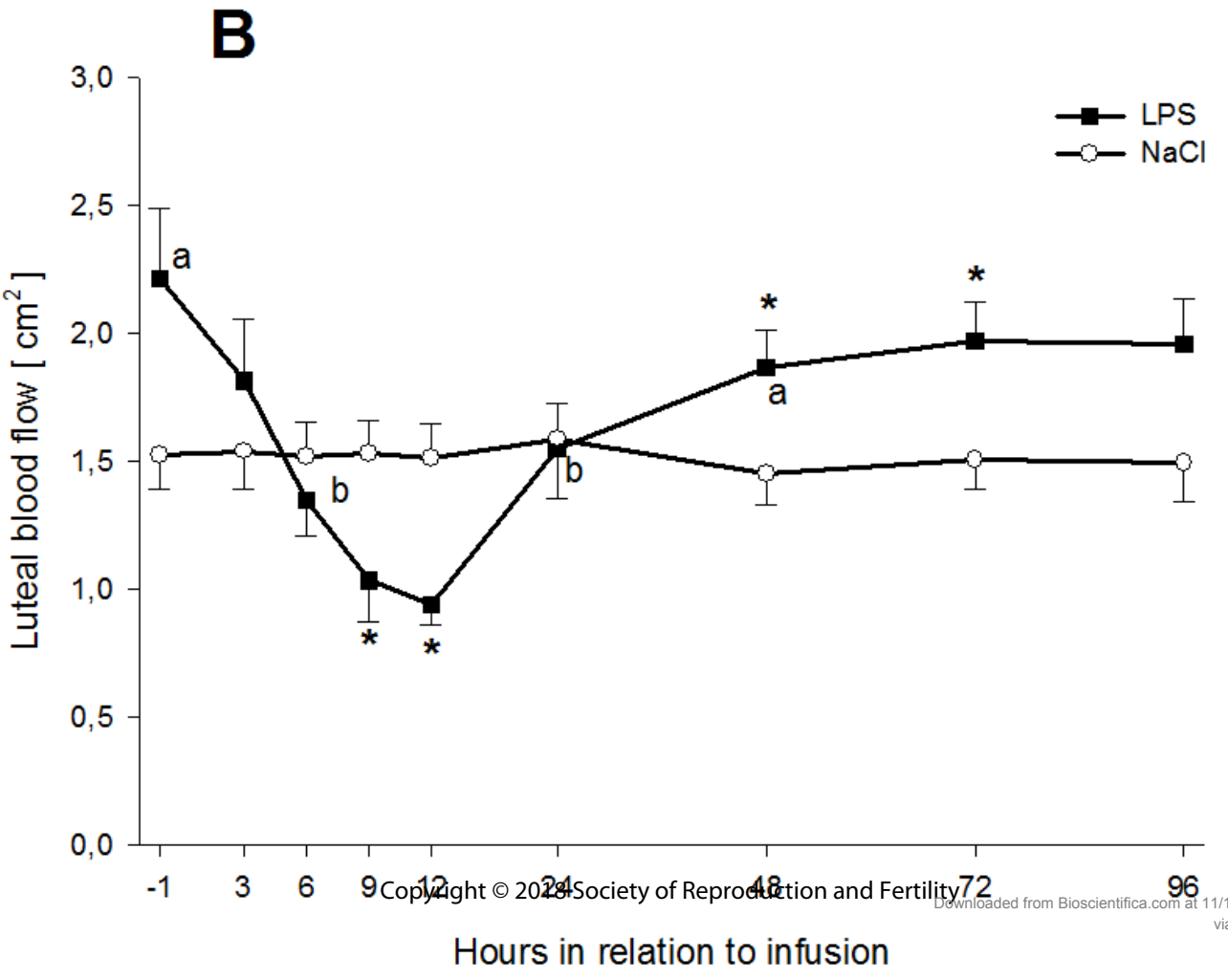
638

639 **Fig. 2:** Median ± MAD of serum progesterone (Panel A) and PGFM concentration (Panel
640 B) in early pregnant cattle treated with *E. coli* lipopolysaccharide (LPS; ▪; -1 to 12 h: n=8,
641 24 to 96 h: n=7) or Saline (○; n=7), respectively.
642 *Values differed between *LPS- and Saline-treated* cattle (P ≤ 0.05).
643 [a,b,A,B]Within *LPS-treated cattle*, values without a common superscript differed (P ≤ 0.05).

644

645 **Fig. 3:** Mean ± SEM levels of mRNA for StAR (Panel A), Casp3 (Panel B), FGF1 (Panel
646 C) and FGF2 (Panel D) 12 and 48 h after infusion of *E. coli* lipopolysaccharide (LPS;
647 dark bars) or *Saline* (NaCl; grey bars) in early pregnant cattle.
648 * Values differed between *LPS- and Saline-treated* cattle within a sampling period (i.e.
649 12 or 48 h), (P ≤ 0.05).
650 [a,b]Within *LPS-treated* cattle, values without a common superscript differed (P ≤ 0.05)





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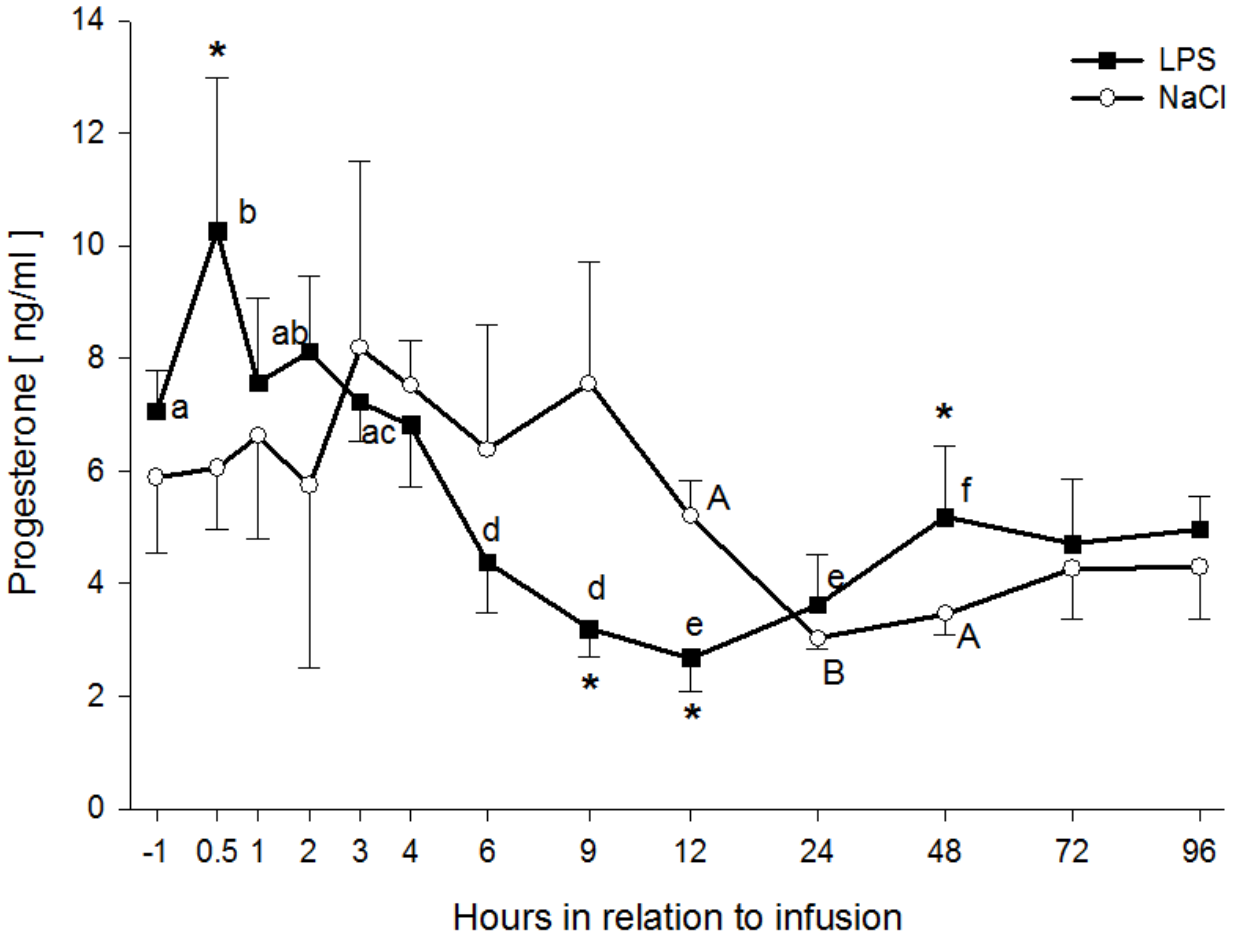
A



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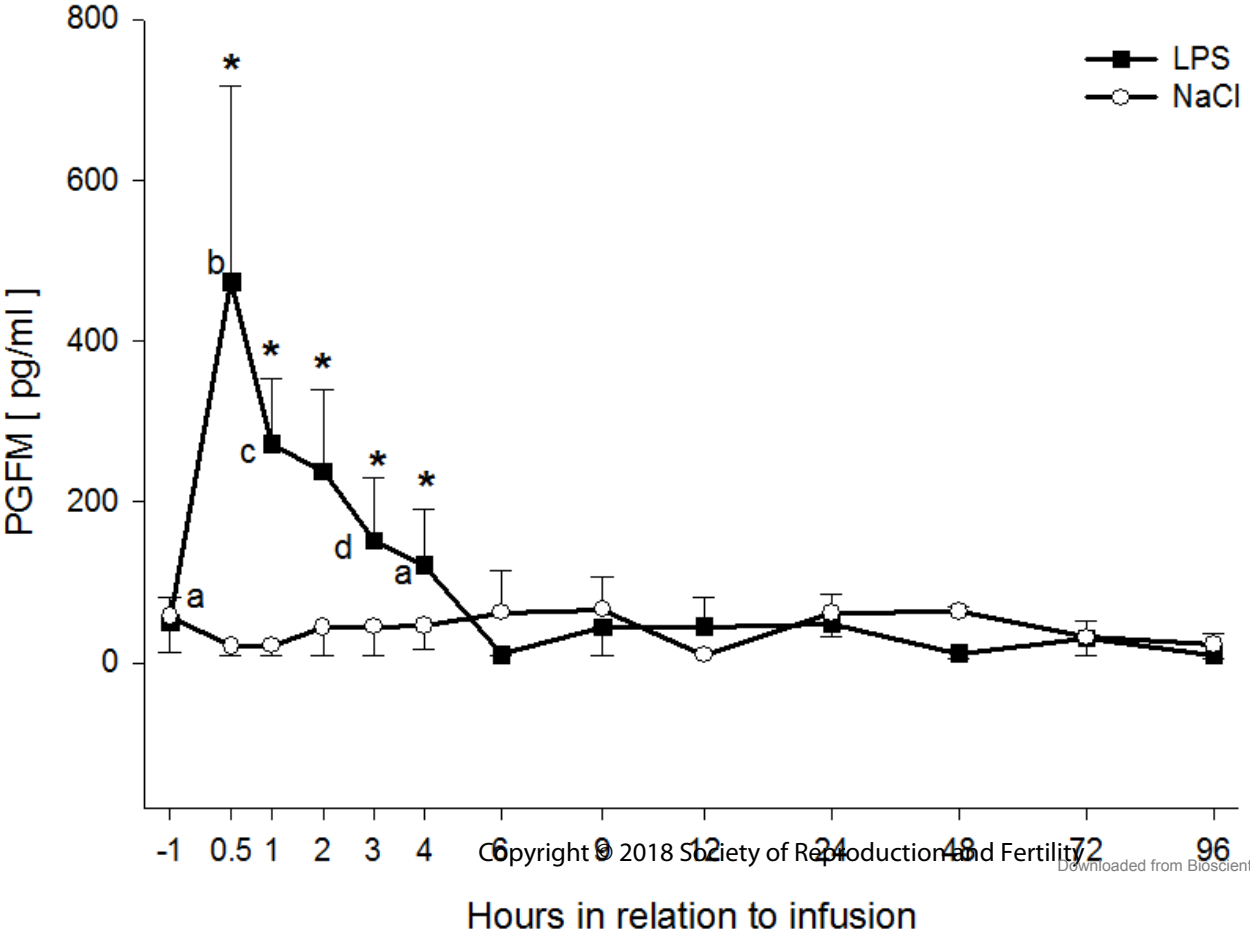

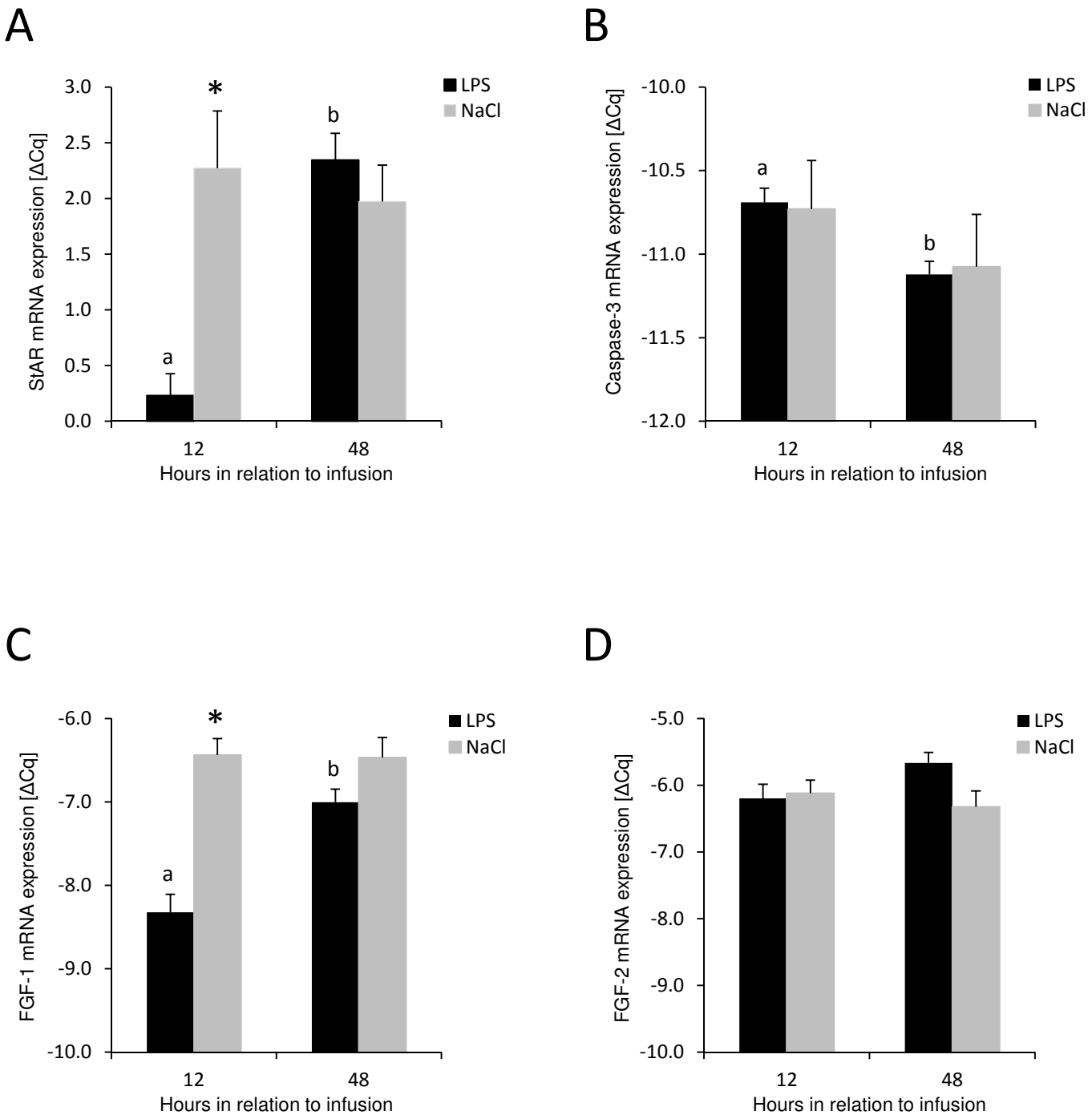

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Gene	Gene symbol	reference [accession no.]	forward primer [5'-…3']	reverse primer [3'…5']	PCR-product [bp]	AT [°C]	FA [°C]	MP [°C]
polyubiquitin	*UBQ3*	NM_174133	AGATCCAGGATAAGGAAGGCAT	GCTCCACCTCCAGGGTGAT	198	60	83	88
histone	*H3F3A*	NM_174133	AGATCCAGGATAAGGAAGGCAT	GCTCCACCTCCAGGGTGAT	233	60	80	87
tyrosine 3-monooxygenase	*YWHAZ*	XM_001927228	AGGCTGAGCGATATGATGAC	GACCCTCCAAGATGACCTAC	141	60	81	87
fibroblast growth factor 1 (acidic)	*FGF1*	NM_174055	GCTGAAGGAGAAACCAGCAC	GTTTTCCTCCAACCTTTCCA	108	60	75	82
fibroblast growth factor 2 (basic)	*FGF2*	NM_174056	TCAAAGGAGTGTGTGTGAAC	CAGGGCCACATACCAACTG	288	60	75	82
toll-like receptor 2	*TLR-2*	NM_174197	CCATGTGGAGAGGGTGTT	GGGGACACAAAACAGCACTT	140	60	81	85
toll-like receptor 4	*TLR-4*	NM_174198	GACCCTTGCGTACAGTTGT	GGTCCAGCATCTTGGTTGAT	103	60	83	88
caspase-3	*CASP-3*	NM_214131	AAC CTC CGT GGA TTC AAA ATC	TTC AGG RTA ATC CAT TTT GTA AC	114	60	75	81
steroidogenic acute regulatory	*StAR*	XR_083945	GGATTAACCAGGTTCGGCG	CTCTCCTTCTTCCAGCCCTC	157	60	84	89

Table 2. Clinical symptoms of each LPS-treated cow. Abbreviations: T= rectal temperature; HR= heart rate; RR= respiratory rate; ESV= episcleral vessels

Time in relation to LPS (hh:mm)		1	2	3	4	5	6	7	8
	-1:00	T 38.6°C HR 76/min RR 24/min No abnormal clinical signs	T 38.4°C HR 56/min RR 36/min No abnormal clinical signs	T 38.7°C HR 56/min RR 24/min No abnormal clinical signs	T 38.5°C HR 64/min RR 24/min No abnormal clinical signs	T 38.5°C HR 72/min RR 32/min No abnormal clinical signs	T 38.6°C HR 60/min RR 24/min No abnormal clinical signs	T 38.8°C HR 72/min RR 40/min No abnormal clinical signs	T 38.7°C HR 56/min RR 24/min No abnormal clinical signs
	0:00 to 0:15	Tachycardia RR to 60/min, eupneic tachypnea Standing, bright, sudden interruption of feed intake	Tachycardia RR to a maximum of 90/min Mild to severe dyspnea, coughing, grunting, stridor Standing, bright Polyuria Epiphora	Standing, bright Tachycardia RR to 52/min Moderate dyspnea, coughing, mild stridor	RR to 36/min Mild dyspnea, coughing Standing, bright	Tachycardia RR to 54/min Mild dyspnea, dilated nostrils, coughing Standing, bright, sudden interruption of feed intake	Standing, bright Tachycardia RR to 56/min Moderate dyspnea, coughing	RR 40/min Standing, bright, nervous	Tachycardia RR 90/min Moderate dyspnea, coughing Standing, becomes less responsive, sudden interruption of feed intake
	0:15 to 0:30	Tachycardia RR to a maximum of 104/min Moderate dyspnea with stridor, sporadic grunting Standing, becomes less responsive, head down	Tachycardia RR 90/min, irregular Moderate to severe dyspnea, frequent and deep grunting Standing, eupneic position (head down and forward, abducted front limbs), anxious, less responsive Eructation 1-2 times per minute	Standing Tachycardia RR to 52/min Moderate dyspnea, coughing, stridor	Tachycardia RR 60 to 80/min Moderate dyspnea with stridor, coughing Standing, becomes less responsive, nervous, head down Ptyalism Epiphora	Tachycardia RR 54 to 104/min Moderate to severe dyspnea with coughing, mouth breathing Standing, eupneic position (holds head forward), nervous, becomes less responsive Ptyalism Mild diarrhea	Standing, bright Tachycardia RR to 100/min Moderate to severe dyspnea with stridor, coughing, mouth breathing	Tachycardia RR to 54/min Moderate dyspnea, coughing Standing, bright, anxious	Standing, bright, anxious Tachycardia RR 90/min Moderate to severe dyspnea with stridor, grunting, coughing, mouth breathing Mild to moderate diarrhea
	0:30 to 1:00	Tachycardia RR to 80/min Moderate dyspnea with stridor, frequent grunting, sporadic coughing Unsteadiness, alternates weight bearing on left vs right rear limbs Ptyalism	Tachycardia RR to 58/min Severe dyspnea with stridor, grunting, coughing Unsteady, alternates weight between rear limbs Ptyalism, epiphora and seromucous nasal discharge	Standing, anxious, unresponsive, holds head down Tachycardia RR to a maximum of 60/min Severe dyspnea with stridor, grunting, coughing Ptyalism Moderate diarrhea	Tachycardia RR 80/min Moderate dyspnea with stridor, grunting, coughing Standing, anxious, unresponsive, ears cold Ptyalism Epiphora and injected ESV Mild diarrhea	Tachycardia RR to 92/min Severe dyspnea with grunting, coughing Standing, nervous, unsteadiness, alternates weight on rear limbs, unresponsive, head down Ptyalism Mild diarrhea	Tachycardia RR to 68/min Moderate dyspnea with stridor, grunting Standing, less responsive, unsteady, alternated weight on rear limbs Ptyalism Moderate diarrhea	Tachycardia RR to 54/min Mild dyspnea, coughing Standing, becomes less responsive, anxious Moderate diarrhea	Tachycardia RR 90/min Moderate to severe dyspnea with dilated nostrils, stridor, grunting, coughing, epistaxis Standing, eupneic position (head down and forward, abducted front limbs), anxious, unsteady, alternates weight between rear limbs, becomes unresponsive
	1:00 to 2:00	T 39.4°C Tachycardia RR 52 to 80/min Moderate dyspnea with stridor Frequent grunting Anxious, drinks repeatedly, Unresponsive Ptyalism	T 38.4°C Tachycardia RR from 24 to 48/min Severe dyspnea with stridor, frequent grunting Anxious, searches repeatedly for water but does not drink, Unresponsive, up and down Ptyalism, epiphora and seromucous nasal discharge Ears cold (only apical) Injected ESV Mild diarrhea	T 38.8°C Tachycardia RR to 48/min Moderate dyspnea with stridor, grunting, coughing Standing, is calm, but still unresponsive Injected ESV Mild diarrhea	T 38.5°C Tachycardia RR 64 to 72/min Severe dyspnea with stridor, grunting, coughing, mouth breathing Up and down, anxious, unresponsive Ptyalism Epiphora and injected ESV Mild diarrhea	T 39.4°C Tachycardia RR 78 to 88/min Moderate dyspnea, stridor, grunting Standing, eupneic position (holds head forward), anxious, unresponsive, alternates weight on rear limbs Ptyalism Injected ESV Tremors	T 38.6°C Tachycardia RR to 64/min Moderate dyspnea, grunting Mostly standing, unresponsive Bristled hair Injected ESV	T = 39.3°C RR 60/min Mild dyspnea, coughing Up and down anxious, unsteadiness, alternates weight between rear limbs, unresponsive, tremors Injected ESV Moderate diarrhea Ptyalism Injected ESV	T = 38.8°C RR 90/min Severe dyspnea Recumbent Moderate diarrhea Tachycardia Injected ESV Hyperemic nasal mucosa
	2:00 to 3:00	T 39.3°C Tachycardia RR 56 to 60/min Moderate dyspnea with stridor, frequent grunting and coughing Up and down Calm, but unresponsive Ptyalism Injected ESV	T 39.0 RR 32/min to 24/min Severe to moderate dyspnea, stridor, grunting sporadically Mostly recumbent, frequently shifted between lying on left vs right Unresponsive Injected ESV Moderate diarrhea Other signs tend to disappear	T 38.8°C Tachycardia RR 44/min to 36/min Moderate to mild dyspnea with stridor Standing, is calm, but still unresponsive, drinks repeatedly Ptyalism disappears Injected ESV Mild diarrhea	T 38.5°C Tachycardia RR 64/min to 54/min Moderate dyspnea with grunting Up and down, calm, but unresponsive Ptyalism Epiphora and injected ESV Mild diarrhea	T 39.0°C Tachycardia RR to 80/min Moderate dyspnea, grunting Standing, calm, but unresponsive, drinks repeatedly Injected ESV	T 38.5°C RR to 64/min Mild dyspnea, grunting Mostly standing, unresponsive Injected ESV	T 39.7°C RR to 60/min Mild dyspnea, stridor, grunting, coughing Recumbent, nervous, unresponsive Moderate diarrhea Ptyalism Injected ESV Tremors	T = 38.8°C RR 90/min Severe to mild dyspnea Recumbent Moderate diarrhea Tachycardia Injected ESV
	3:00 to 6:00	T 38.6°C Tachycardia gradually decreases RR slowed from 56 to 36/min (3:00 to 5:00) Moderate to mild dyspnea with gurgling and stridor; grunting and cough disappear gradually 5:00 becomes calm and responsive Ptyalism Injected ESV 5:00 to 6:00 polyuria	T 38.5°C RR 27/min Mild dyspnea tends to decrease, slight stridor, grunting sporadically Mostly standing, is calm, but still unresponsive Injected to normal ESV Mild diarrhea	T 38.4°C 3:00 No tachycardia RR 36/min to 20/min Mild dyspnea tends to disappear Up and down, becomes calm and responsive Tremors Injected ESV Mild diarrhea	T 38.2°C Tachycardia gradually decreases RR 40 to 48/min Moderate to severe to mild dyspnea with stridor, grunting, coughing, gradually decreasing Up and down, head down, unresponsive Tremors Ptyalism Injected ESV Mild diarrhea	T 37.5°C (4:17) to 38.0°C (5:00) No tachycardia (4:17) RR to 56/min Mild dyspnea, coughing, grunting Up and down, still unresponsive Mild dehydration Tremors Injected ESV	T 39.3°C RR to 56/min Mild dyspnea, grunting Mostly standing, becomes calm and responsive (4:53) Injected ESV	T 39.4°C (3.06) to 39.0 (4.08) RR to 44/min Mostly recumbent, becomes calm and responsive All signs progressively disappear	T = 38.8°C Tachycardia RR 90/min Moderate dyspnea decreases to disappearing (3:53) Mostly standing, becomes calm and bright (3:53) Moderate diarrhea Tremors All signs tend to disappear 5:50 T, HR and RR in normal range, no abnormal clinical signs
	6:00 to 12:00	T 38.4°C RR 36/min Mild dyspnea and stridor gradually disappeared 6:00, feed and water intake restored 7:50 all signs disappeared	T 38.5°C 8:51 HR and RR in normal range, no abnormal clinical signs	T 38. 6°C Mostly recumbent, calm, bright RR 24/min 07:25 no abnormal clinical signs	T 38.3°C RR 40/min No dyspnea Mostly recumbent 7:15 becomes calm and responsive, feed and water intake restored All other signs gradually disappear 9:45 no abnormal clinical signs	T 38.7°C RR 44 to 58/min Mild dyspnea only when recumbent, cough Mostly recumbent, unresponsive, feed and water intake restored Injected ESV All other signs disappear (11:17)	T 39.3°C RR to 40/min Mild dyspnea, then decreased Mostly recumbent, bright, feed and water intake restored (6:00) 9:03 no abnormal clinical signs	T 38.8°C Mostly recumbent, bright 6:06 HR and RR in normal range, no abnormal clinical signs	T = 38.8°C HR and RR in normal range, no abnormal clinical signs Up and down, bright
	12:00 to 24:00 (end)	12:00 T, HR and RR in normal range, no abnormal clinical signs	12:00 T, HR and RR in normal range, no abnormal clinical signs 21:00 Found dead (apparently died between 15:00 and 20:00)	12:00 T, HR and RR in normal range, no abnormal clinical signs	12:00 T, HR and RR in normal range, no abnormal clinical signs	12:00 T 38.4°C RR 40/min No dyspnea Standing, slightly unresponsive Injected ESV 24:00 T, HR and RR in normal range, no abnormal clinical signs	12:00 T, HR and RR in normal range, no abnormal clinical signs	12:00 T, HR and RR in normal range, no abnormal clinical signs	12:00 T, HR and RR in normal range, no abnormal clinical signs